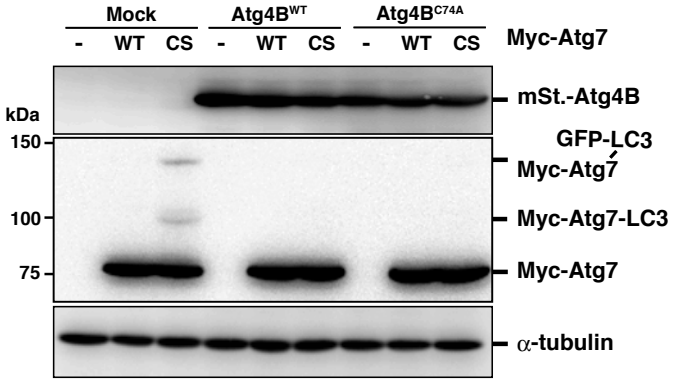
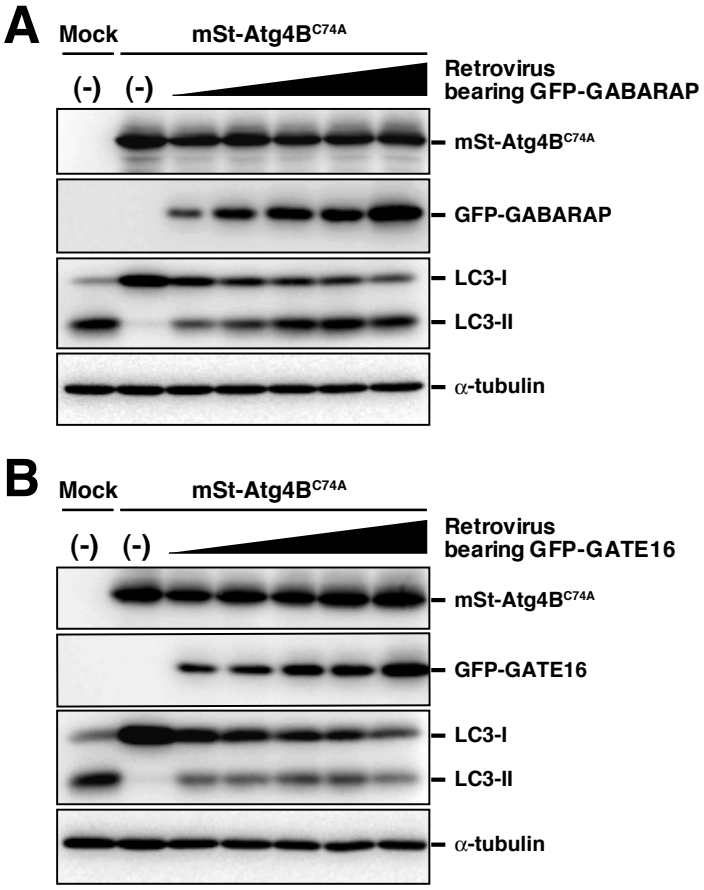


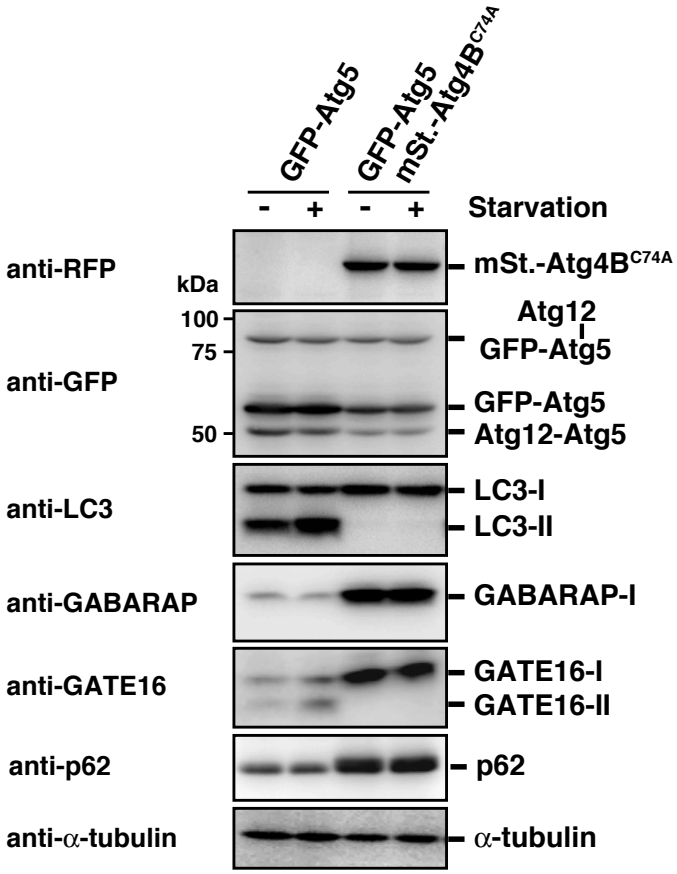
Supplemental Figure S1. Overexpression of wild-type Atg4B, as well as inactive Atg4B mutant, sequesters the LC3 from the access to Atg7.
MCF7 cells stably expressing GFP-LC3 were infected with combination of recombinant adenoviruses as indicated. After 40 hours incubation, the cells were lysed and the samples were examined by Western blotting using each antibody. From top panel, anti-RFP, anti-Myc, anti-α-tubulin.

Fujita et al. Figure S1



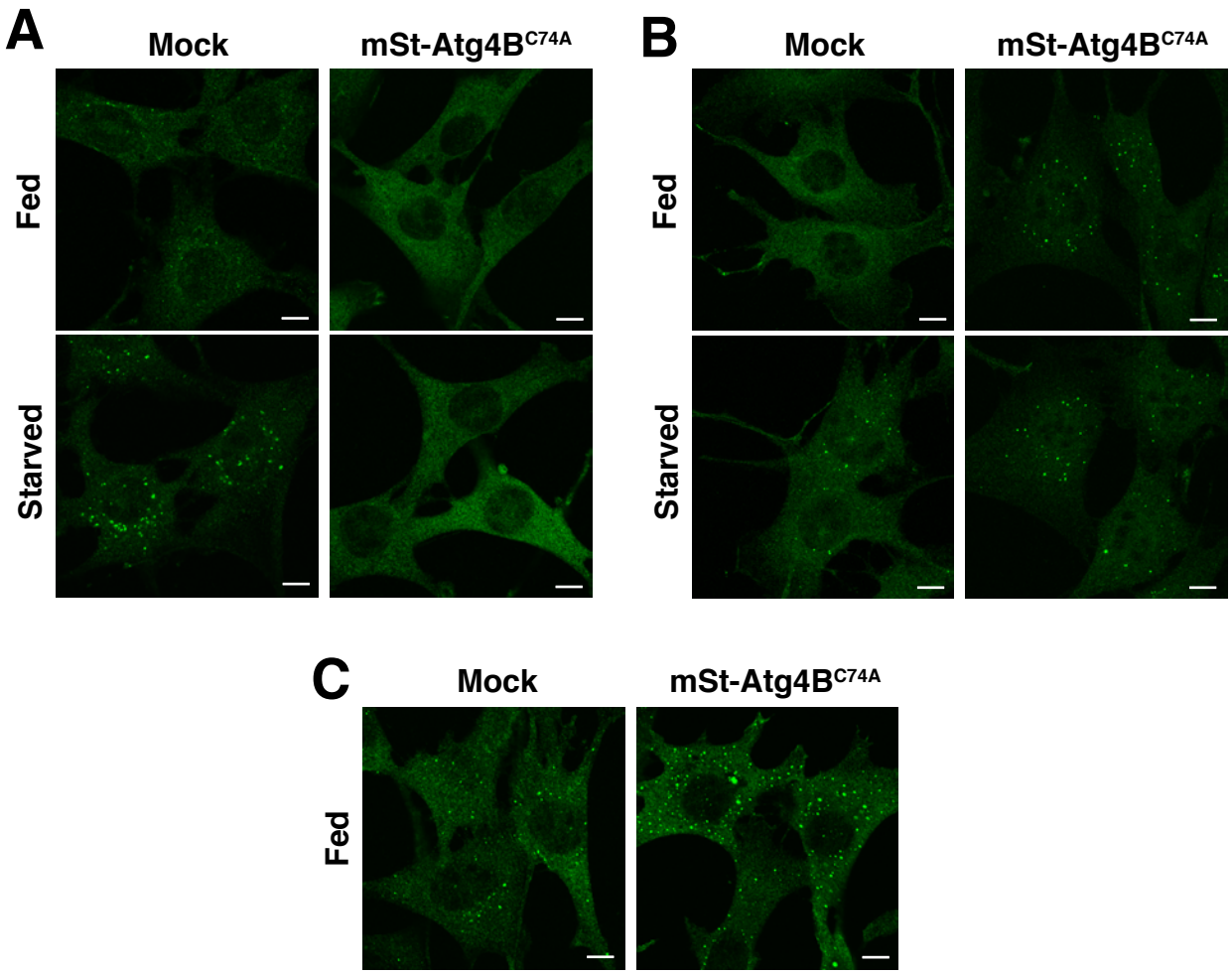
Supplemental Figure S2. The inhibitory effect of Atg4B mutant is suppressed by exogenous mammalian Atg8 homologues in a dose-dependent manner.
NIH3T3 cells stably expressing mStrawberry-Atg4B^{C74A} were infected with different amounts of retroviruses bearing GFP-GABARAP (A) or GFP-GATE16 (B) and then stable transformants were selected in growth medium with 10 μg/ml blastcidin. Parent NIH3T3 cells and the stable transformants were cultured in HBSS (Starved) for 1 hour, and cell lysates were examined by Western blotting using each antibody. From top panel, anti-RFP, anti-GFP, anti-LC3, anti-α -tubulin.

Fujita et al. Figure S2



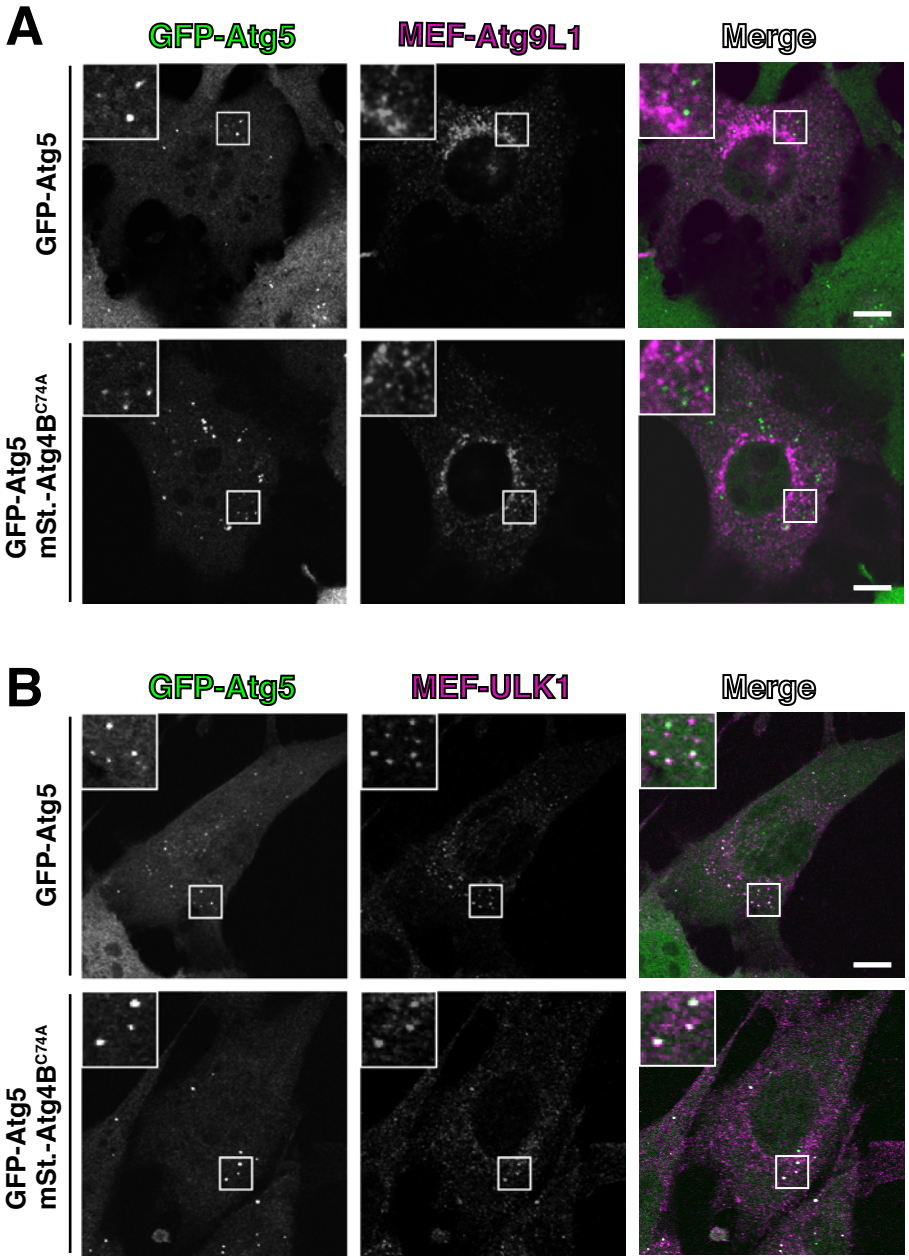
Supplemental Figure S3. Effect of Atg4B^{C74A} overexpression on PE conjugation of LC3 paralogues in NIH3T3 cells.
NIH3T3 cells stably expressing GFP-Atg5 or both GFP-Atg5 and mStrawberry-Atg4BC74A were cultured in growth medium or HBSS for 1-h. Cell lysates were examined by Western blotting using the indicated antibodies.

Fujita et al. Figure S3



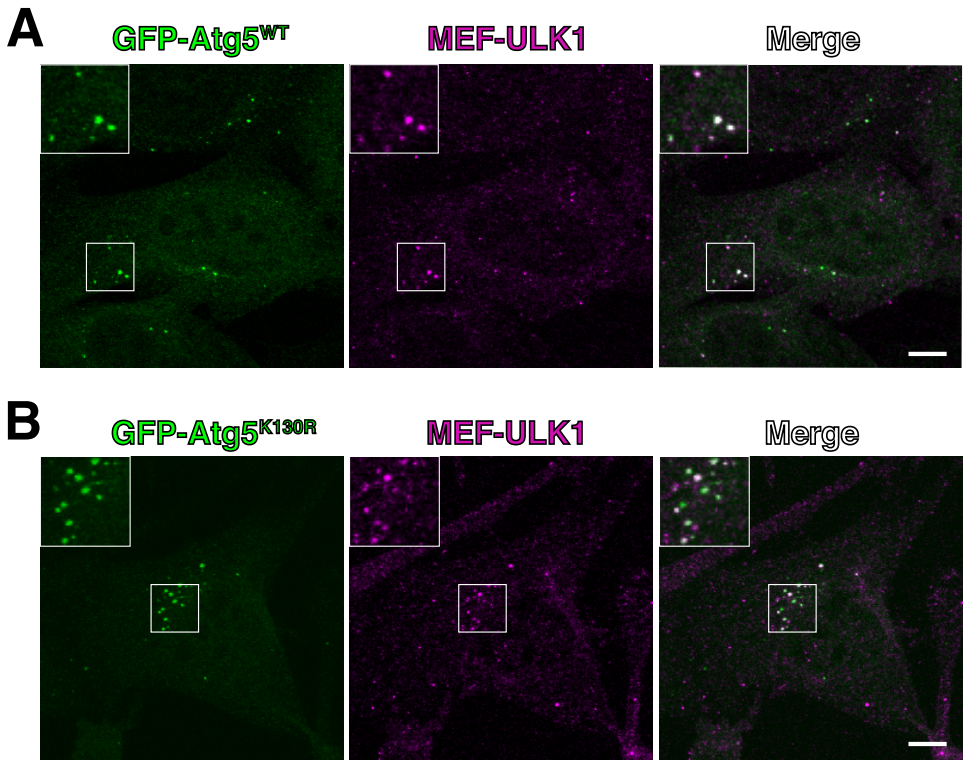
Supplemental Figure S4. Effect of Atg4B^{C74A} overexpression on localization of LC3, Atg16L, and p62 in NIH3T3 cells.
NIH3T3 cells stably expressing empty vector or mStrawberry-Atg4B^{C74A} were cultured in growth medium (Fed) or HBSS (starved) for 1 hour, fixed, and subjected to immunocytochemistry using anti-LC3 (A), anti-Atg16L (B), or anti-p62 (C) antibody. Anti-rabbit antibody conjugated with Alexa488 was used as secondary antibody. Bar indicates 10 μm.

Fujita et al. Figure S4



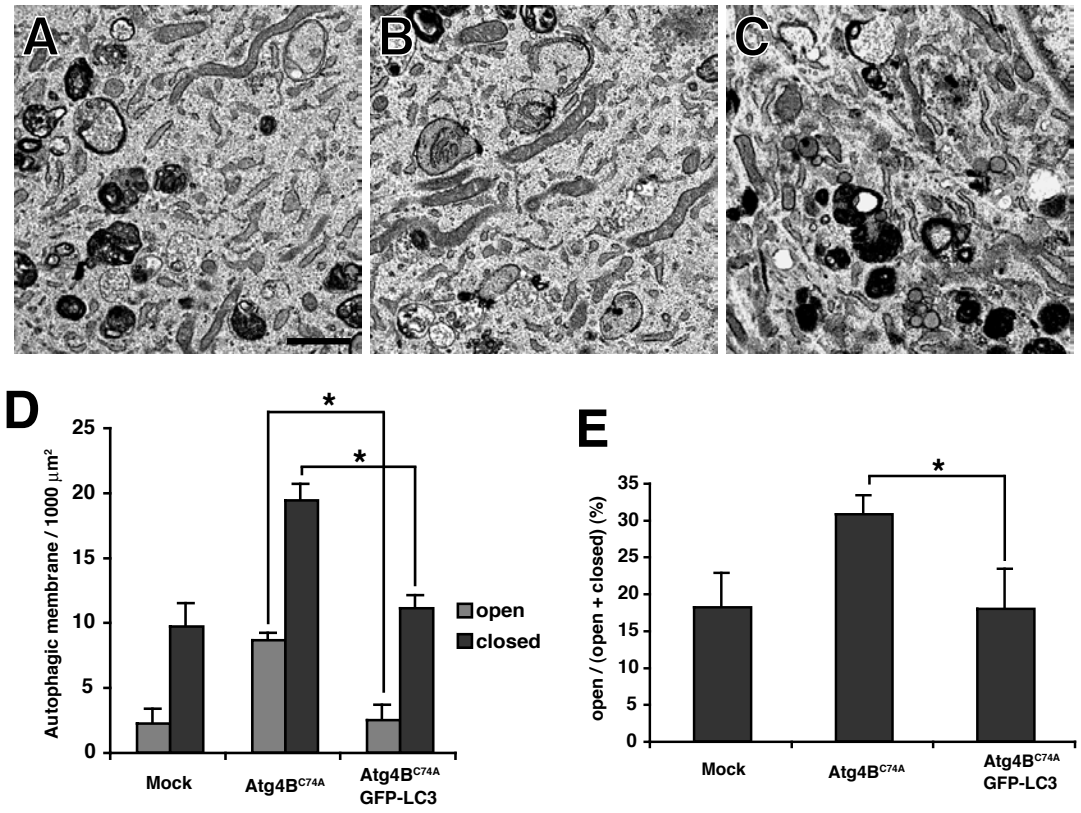
Supplemental Figure S5. ULK1 localizes on Atg5-positive membranes in NIH3T3 cells stably expressing mStrawberry-Atg4B^{C74A}.
NIH3T3 cells stably expressing GFP-Atg5 or both GFP-Atg5 and mStrawberry-Atg4B^{C74A} were transfected with myc-TEV-Flag-tagged-mAtg9L1 (A) or -ULK1 (B). After 40-h incubation, the cells were cultured in HBSS for 1 h, and fixed. The cells were labeled with mouse anti-myc monoclonal antibodies and Alexa647-conjugated anti-mouse antibodies, and were observed by confocal laser scanning microscopy (FV1000, Olympus). Bar indicates 10 μm.

Fujita et al. Figure S5



Supplemental Figure S6. ULK1 was recruited to the GFP-Atg5-positive structure independent of Atg12-Atg5 conjugation.
Atg5 knockout MEF cells stably expressing myc-TEV-Flag-tagged ULK1 and GFP-Atg5^{WT} (A) or myc-TEV-Flag-tagged ULK1 and GFP-Atg5^{K130R} (B) were cultured in HBSS for 1 hour, fixed, and subjected to immunocytochemistry using anti-myc antibody. Anti-mouse antibody conjugated with Alexa568 was used as secondary antibody. Bar indicates 10 μm.

Fujita et al. Figure S6



Supplemental Figure S7. The effect of GFP-LC3 overexpression in the mStrawberry -Atg4B^{C74A} expressing cells.
(A-C) NIH3T3 cells stably expressing empty vector (Mock) (A), mStrawberry -Atg4B^{C74A} (B), or both mStrawberry- Atg4B^{C74A} and GFP-LC3 (C) were cultured in HBSS for 1 h, fixed, and subjected to conventional electron microscopic analysis. Representative images are shown. Bar indicates 1 μm. (D) The number of autophagic structures in mock, mStrawberry-Atg4B^{C74A}, or both mStrawberry-Atg4B^{C74A} and GFP-LC3 expressing cells. Open: open autophagic membranes. Closed: closed autophagic membranes. (E) The ratio of the number of open structures to total autophagic structures. Data are the means ± standard deviation of duplicates from representative experiments. Asterisks indicate a significant difference, $p<0.05$

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Supplemental movies 1 and 2.

NIH3T3 cells stably expressing GFP-Atg5 (1) or both GFP-Atg5 and mStrawberry-Atg4B^{C74A} (2) were grown in HBSS for 1 h and directly observed by time-lapse video microscopy.